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The Laboratory of Molecular Genetics (LMG) investigates the molecular basis for aging and age-dependent diseases, notably cancer. Studies focus on DNA related mechanisms such as genomic instability, DNA repair, DNA replication, and transcription. We consider the increased DNA damage accumulation in senescence as the major molecular change with aging, and this DNA damage may eventually inactivate individual genes and lead to a deterioration of the organism which is characteristic of the senescent phenotype. The goal of LMG is thus to understand the underlying mechanisms involved in DNA damage formation and its processing as well as the changes that take place with aging and that make aging cells susceptible to cancer. DNA repair is likely to play a critical role, and we have a special interest in the fine structure of DNA repair which includes the study of the DNA repair processes in individual genes. We are investigating the molecular mechanisms involved in DNA repair and in genomic instability in normal, senescent and cancer cells. We are studying the molecular biochemistry of the DNA repair processes nucleotide excision repair and base excision repair in *in vitro* systems, in fractionated cell extracts, and in intact cells. We are also interested in the molecular processes that interact with the DNA repair processes. They include transcription, replication, somatic mutation and mitochondrial alterations.

The accumulation of DNA damage with age could be a result of a gradual decline in DNA repair capacity. Work from this and other laboratories suggests that this decline is not readily detectable in the overall genome, but may rather be a decline in the fine structure or transcription coupled component of the DNA repair process.

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Orren D, et al. *Mol Biol Cell* 1997; 8: 1129-1142.

Croteau DL et al. *J Biol Chem* 1997; 272: 25409-25412.

Biography: Dr. Bohr received his M.D. in 1978, Ph.D. in 1987, and D.Sc. in 1987 from the University of Copenhagen, Denmark. After residencies in neurology and infectious diseases at the University Hospital in Copenhagen, Dr. Bohr did a postdoctoral fellowship with Dr. Hans Klenow at the University of Copenhagen. He then worked with Dr. Philip Hanawalt at Stanford University as a research scholar during 1982-1986. In 1986 he was appointed to the National Cancer Institute (NCI) as an investigator, becoming a tenured Senior Investigator in 1988. Dr. Bohr developed a research section in DNA repair at the NCI. In 1992 he moved to the NIA to become Chief of the Laboratory of Molecular Genetics.

Dr. Bohr has conducted clinical studies (infectious diseases and oncology), but worked most extensively in basic research. His main contributions have been in the area of DNA repair. He has worked on many aspects of DNA damage and its processing in mammalian cells. He developed a widely used method for the analysis of DNA repair in individual genes and found that active genes are preferentially repaired. This observation was a major advance in the clarification of the tight interaction between DNA repair and transcription, a process termed transcription-coupled repair. In recent years numerous papers from his laboratory have focused on mechanisms of DNA damage processing, particularly on nucleotide excision repair and transcription coupling. A main interest now is to elucidate those processes in relation to aging.

DNA Repair Processes: Several types of DNA lesions have been observed in mammalian DNA and they are removed by a number of different DNA repair pathways. One important pathway is nucleotide excision repair (NER), which removes and replaces bulky lesions, such as UV-light induced pyrimidine dimers. Damaged bases are removed as nucleotides, typically as oligonucleotide fragments. This pathway involves several of the xeroderma pigmentosum DNA repair proteins. Another important DNA repair pathway is base excision repair (BER), which removes single damaged bases as free bases, and replaces them. Base excision repair removes a large number of minor lesions from DNA, many that had been caused by oxidative modification. A third important pathway of DNA repair is mismatch repair, which occurs during DNA replication. Finally, a fourth pathway is recombination repair.

In the Laboratory of Molecular Genetics we mainly focus on NER and BER. We are interested in some of the subcomponent DNA pathways: gene-specific DNA repair and transcription-coupled repair (TCR). TCR reflects the tight interaction between DNA repair and transcription that leads to the highly efficient removal of lesions from the transcribed strand of active genes. Gene-specific DNA repair occurs at the nuclear matrix, where a number of repair proteins are recruited early in the repair process.

Transcription-Coupled Repair: Research in TCR has recently become a very fast moving area. There has been excitement about the discovery of a close molecular linkage between the processes of DNA transcription and repair. The most dramatic proof of this linkage was the finding a few years ago that the basic transcription factor, TFIIH, which is involved in transcription of DNA into mRNA, contains at least two DNA repair proteins among its seven subunits. The linkage between transcription and repair is now understood to be so close that they overlap extensively. Much evidence supports the idea that the TCR pathway and the gene-specific DNA repair pathway are different from the DNA repair pathways that operate in the rest of the genome, which consists of transcriptionally inactive bulk DNA.

Despite the interest in TCR, questions about the exact mechanism remain unanswered: What is the signal for TCR? Is the signal a RNA polymerase that is stalled at a lesion? What is the signal from the transcription complex to the repair proteins? Which lesions are repaired by TCR? Can oxidative DNA damage, thought to accumulate with aging, be repaired by TCR? To answer these questions about the mechanism of TCR, we are taking a number of approaches. DNA repair is studied in intact cells, in situ, in tissue culture, in cell extracts, or using purified components. DNA damage is induced by exposure of cells or purified DNA to various types of DNA damage or cellular stress.

DNA Repair and Aging: DNA damage is observed to accumulate with senescence. Subtle defects in DNA repair that arise during aging could explain this observation. The question of whether or not DNA repair declines with aging has been of great interest, and is a central concern of our research. We have many projects that address this issue. Whereas there is no hard evidence for a decline in the efficiency of the general genomic, DNA repair during aging, there is some evidence for a decline in the DNA repair fine structure: gene specific repair, or TCR. This is currently being investigated.

Oxidative DNA Damage and Mitochondrial Functions: Reactive oxygen species are generated in cells as a by-product of cellular metabolism. It is a main product of the metabolic processes in each cell, and reactive oxygen species react with proteins, lipids, and DNA. Oxidative DNA damage is thought to be more related to aging than is any other form of DNA damage, and it has been shown to accumulate with aging. Oxidative DNA damage results from various forms of cellular stress, such as through the action of hydrogen peroxide generated intracellularly, and through exposure to acridine orange, X-irradiation, or methylene blue. Oxidative damage is thought to contribute to carcinogenesis, mitochondrial dysfunction, and aging.

Because most reactive oxygen species are generated by the oxidative phosphorylation processes that occur in mitochondria, it is of great interest to understand the oxidative DNA damage processing mechanisms in mitochondria. Mitochondrial DNA is not protected by histones and lies in close proximity to the free radical producing electron transport chain. Mitochondrial DNA contains a higher steady state amount of oxidative DNA damage than nuclear DNA. Oxidative DNA damage that arises in mitochondrial DNA might give rise to the mutations, gene inactivations, or deletions that are commonly found in the mitochondrial genome in association with aging and cancer. Because mitochondrial DNA is subjected to relatively higher amount of oxidative damage, it seems that mitochondria would need DNA repair activity to remove oxidative damage from their DNA. In the absence of DNA repair, mitochondrial DNA mutations would arise at high frequencies. Mitochondria are known to be defective in DNA repair, particularly in nucleotide excision repair. Mitochondrial DNA mutations and deletions accumulate in the elderly and in patients with mitochondrial myopathies. Whether these arise as a result of defective DNA repair remains to be explored.

The early finding of absence of repair of UV-induced pyrimidine dimers in mitochondrial DNA led to the general notion that there is no DNA repair in mitochondria. More recently, DNA repair enzymes have been identified from mitochondria, and gene specific repair experiments have shown efficient repair in mitochondrial DNA. We demonstrated removal of cisplatin interstrand cross-links, N-methylpurines, 8-oxo-G, and some removal of cisplatin intrastrand crosslinks in mitochondrial DNA sequences. These and more recent studies from our group and elsewhere have shown that a number of lesions are efficiently repaired from mitochondrial DNA.

Oxidative DNA Damage and Repair in Nuclear and Mitochondrial DNA: When DNA is the target of oxidative stress, a variety of DNA adducts can be formed, of which 8-hydroxyguanine (8-oxo-G) is one of the most abundant lesions generated. 8-oxo-G is thought to be a pre-mutagenic lesion because it can mispair with adenine during DNA replication, and this mispairing results in G-T transversion mutations. Although cells use a combination of base excision repair and nucleotide excision repair to remove lesions generated by oxidative damage, base excision repair is the most important.

We have developed a technique to measure the formation of 8-oxo-G in individual genes. We have shown that this lesion is efficiently repaired both in nuclear and mitochondrial DNA.

We can also measure the repair of oxidative damage *in vitro*. Results based on the use of mutant cell lines and on enzyme inhibitors suggest that nucleotide excision repair is involved in the removal of 8-oxo-G lesions. This is supported by results of experiments with xeroderma pigmentosum (XP) cells. We used XP cells that are completely defective in nucleotide excision repair but are proficient in base excision repair. 8-oxo-G lesions are not removed as efficiently in XPA cells as they are in normal cells. This result suggests that nucleotide excision repair is involved in repair of 8-oxo-G lesions. We are interested in further exploring the interaction between nucleotide excision repair and base excision repair. The repair of oxidative lesions is being studied in various natural human mutant cell lines suspected or known to be deficient in DNA repair.

DNA Repair in Mitochondria: Studies on mitochondrial DNA damage and repair have traditionally required the purification of mitochondrial DNA. This purification is laborious, and in addition, it is possible that most purification schemes introduce oxidative lesions in the DNA. As an alternative approach, we modified the gene specific repair assay that we had developed to detect various DNA lesions other than UV-induced pyrimidine dimers. With this approach, we do not need to isolate mitochondrial DNA, and can probe for oxidative lesions in the entire mitochondrial genome or in parts of it. We have established an assay using a repair enzyme that detects 8-oxo-G. This lesion is repaired very efficiently from both mitochondrial and nuclear DNA.

We have partially purified a mitochondrial oxidative damage endonuclease (mt ODE) from rat liver that recognizes and incises 8-oxo-G and abasic sites in duplex DNA. The name reflects that the enzyme incises apurinic/

apyrimidinic (AP) sites. Comparison of mt ODE with other known 8-oxo-G glycosylases/abasic lyases and mitochondrial enzymes reveals that this is a novel protein with similarity to the OGG1 enzyme from yeast, the gene for which was recently cloned. To the best of our knowledge, this is the first characterization of a mammalian mitochondrial enzyme that recognizes oxidative DNA damage.

We have measured mt ODE's incision activity from 6- and 24-month old rat liver. Interestingly, there is no decline, but instead an increase in activity with age. This finding is contrary to current notions of mitochondrial decline and is being pursued further experimentally.

We are also establishing experimental conditions for the study of DNA repair in mitochondrial extracts. For example, we have developed an assay for DNA nicking activity in mitochondrial extracts from rats. The assay can detect nicking activity on plasmids containing different types of DNA damage. We plan to determine which of the different types of lesions are recognized in mitochondria as a way to better understand which DNA repair pathways operate in these organelles. A particular focus is whether there are any nucleotide excision repair or recombinational repair pathways. Mitochondrial repair studies have suffered from a lack of availability of *in vitro* systems for biochemical study. We are now purifying components and antibodies to many proteins involved in nucleotide excision repair and base excision repair, and these will be tested for their effect on mitochondrial DNA incision. We will determine whether the mechanism of mitochondrial DNA repair differs from that of nuclear DNA repair, whether mitochondrial DNA repair declines with age, and whether local DNA repair defects in mitochondria lead to DNA deletions.

Quantitation of Oxidative DNA Damage: One of the controversies in the study of oxidative DNA damage concerns the validity of current methods of quantitation of the amount of 8-oxo-G in nuclear and mitochondrial DNA. In general, the amounts measured by various methods (gas chromatography/mass spectroscopy analysis; HPLC; enzymatic analysis) do not agree with one another, and different methods have not been directly compared in the same system.

In collaboration with Dr. Miral Dizdaroglu at National Institute of Standards and Technology, we are using various assays to compare the concentrations of levels of 8-oxo-G in nuclear and mitochondrial DNA. Formation of 8-oxo-G is one of about 100 base changes seen after exposure of cells to oxidative stress, but it is the one for which we have the best analytic tools. Given a sufficiently large DNA sample, however, it is possible to use gas chromatography/mass spectroscopy analysis to quantitate a variety of oxidative lesions. Preliminary results indicate that the concentration of some lesions decreases with age, whereas the concentration of others increases. Many laboratories reported 10-fold higher steady state concentrations of level 8-oxo-G in mitochondrial DNA than in nuclear DNA. This finding has become one of the cornerstones of the mitochondrial theory of aging, but other observations suggest that it may not be true for cells in culture. We are currently further investigating the formation of 8-oxo-G in mitochondrial DNA. By testing different mitochondrial purification schemes, we are exploring whether 8-oxo-G formation is an artifact of mitochondrial isolation techniques.

Changes in Mitochondrial Function with Aging: In Dr. Richard Hansford's section, work on mitochondria isolated from rat heart showed that cardiac mitochondrial cytochrome oxidase (COX) activity decreases 30% with aging. By contrast, the activity of the nuclear-encoded enzyme citrate synthetase does not decline with age, when measured in the same mitochondrial suspensions. This supports the possibility of a specific decline in synthesis of mitochondrial DNA-encoded proteins during aging. All of the COX polypeptides, including the COX I, COX II, and COX III subunits, were markedly reduced in preparations from senescent rats. Western analysis showed a 25% decrease in the concentrations of the COX I polypeptide, but no change in the nuclear-encoded COX IV subunit, supporting the view that there is a specific decrement in the expression of the mitochondrial genome with aging. Northern blot analysis showed a marked decrease in COX I and COX II mRNA isolated from freeze-clamped hearts of old animals compared with young ones. As with protein concentrations, mRNA from nuclear-encoded COX IV and other nuclear-encoded mRNAs showed no age-linked decrease. These results suggest that mitochondrial DNA transcription is less active in the aging rat heart.

Substrates for DNA Repair Studies: DNA repair assays are done mostly with UV-damaged DNA, and sometimes with DNA damaged by cisplatin. UV-damaged DNA and cisplatin-damaged DNA can be repaired by nucleotide excision repair. However, for a number of our assays, we needed to have oligodeoxyribonucleotides or plasmid constructs that contain single lesions. We now have single-lesion plasmid constructs containing oxidative-damage sites or pyrimidine dimers, situated either on the transcribed or on the coding strand.

Premature Aging Syndromes: A number of rare mutations and disorders in humans are associated with premature aging. The patients prematurely have some signs and symptoms associated with normal aging. However, these syndromes are segmental in that some of the features noted in normal aging are not found in the patients.

We are particularly interested in Cockayne syndrome (CS) and in Werner syndrome (WS), which we believe represent optimal model systems for molecular studies of normal human aging. The *WRN* gene, which causes WS, has been recently cloned. The *WRN* gene, the *CS* gene, and other genes mutated in premature aging syndromes encode putative helicases. Therefore, further understanding of the molecular defects in these disorders is a high and achievable priority in the understanding of normal aging. The functions of the CSB protein, which is associated with CS, and of the WRN protein, which is associated with WS, appear to be at the crossroads of aging, DNA transcription, replication, and repair, thereby nicely affording a combination of our interest in DNA function with our interest in aging.

Transcription and DNA Repair in Cockayne Syndrome Cells: Cockayne syndrome (CS) is a rare human disease that is characterized by arrested postnatal growth and other features, resulting in premature aging and death. Cells from CS patients are abnormally sensitive to UV light and to chemicals that mimic the action of UV light. CS cells exhibit delayed recovery of DNA and RNA synthesis after UV irradiation. In normal cells, DNA repair of damaged, transcriptionally active genes occurs faster than DNA repair of inactive parts of the genome. Furthermore, damage in template strands of active genes is normally repaired faster than is damage in the coding strand. CS cells are, however, defective in the preferential repair of active genes, and in strand-specific repair. Complementation studies demonstrated at least two genes, designated *CSA* and *CSB*, are involved in CS.

The complex clinical phenotype of CS, however, suggests that DNA repair may not be the primary defect. Moreover, recent evidence from our laboratory demonstrated that intact or permeabilized CSB cells are defective in RNA polymerase II (Pol II) transcription. Furthermore, we compared Pol II transcription in extracts from CS cells with transcription in extracts prepared from normal cells. We found that Pol II transcription in extracts of CS cells is highly sensitive to minor damages in template DNA arising during purification. This deficiency could be complemented by transfection of a CSB cell line with a normal *CSB* gene. These results support the notion that reduced gene-specific repair in CS is a consequence of a transcription deficiency. Clearly, however, further studies are needed to determine the molecular basis of CS.

Werner Syndrome: Werner syndrome (WS) is a homozygous recessive disease characterized by early onset of many characteristics of normal aging, such as wrinkling of the skin, graying of the hair, cataracts, diabetes, and osteoporosis. A hallmark defect in WS is genomic instability characterized by karyotypic abnormalities including inversions, translocations, and chromosome losses.

The molecular basis of genomic instability in WS remains to be defined. Our laboratory is using several approaches to identify and characterize the molecular defect in WS cells. One approach is to compare the DNA metabolic activities of WS and normal cells. WS cells are not more sensitive to treatment with DNA damaging chemicals, and do not have defective DNA repair after treatment with these chemicals. However, some WS cells appear to have a subtle defect in TCR and they may also have a lower transcription rate than normal cells. This question is currently under active investigation. Experiments with cell extracts suggest that the decrease in transcription rate is due to the presence of an inhibitor of RNA polymerase II (Pol II) in WS cells which could also explain the defect in TCR.

Cells from WS patients grow more slowly and become senescent at an earlier population doubling than age-matched normal cells, possibly because the WS cells appear to have accelerated losses of the telomeric ends of their chromosomes. Telomeric shortening is an established marker of cellular senescence.

We are also examining the slow growth characteristics of WS cells, emphasizing on determining whether this truly reflects accelerated cellular senescence. Established markers of cellular senescence, such as telomere length and ß-galactosidase activity, are being compared in cultured WS cells and normal cells. We also plan to determine if the cell cycle is disrupted in WS cells.

We are also purifying WRN protein for use in a number of basic and complex biochemical assays. Once purified to homogeneity, the WRN protein will be examined for its activities and potential interactions with other proteins.

Immunology and DNA Repair: After antigenic stimulation, genes encoding the variable regions of antibodies undergo somatic hypermutation, contributing to antibody diversity. Dr. Patricia Gearhart's unit is investigating whether DNA repair processes play a role in somatic hypermutation.

We examined a number of knockout mice for DNA repair genes. The results convincingly showed a change in the pattern of somatic hypermutation in mismatch repair-deficient mouse strains. These results suggest that DNA repair processes and DNA polymerase fidelity both play a role in somatic hypermutation. We are also identifying DNA sequences in the variable region that focus the hypermutation on to the gene. To study the mechanism itself, we are looking at the expression of various errorprone DNA polymerases in B lymphocytes..

Breast Cancer: Dr. Michele Evans is exploring the efficiency of DNA repair in various forms of breast cancer. Some previous studies have indicated a possible DNA repair defect in individuals with breast cancer and their relatives. Our current explorations involve gene specific DNA repair measurements and also the levels of expressions of repair genes in hormone dependent and independent breast cancer cell lines. We plan to also investigate the DNA repair in old and young breast cancer patients using the cohort in the Baltimore Longitudinal Study of Aging.

Cell Cycle Progression and DNA Damage Processing: We have been interested in the relationship between DNA damage processing and cell cycle regulation. We analyzed the effect of DNA damage on the progression of the cell cycle in synchronized Chinese hamster ovary (CHO) cells. We also measured the extent of TCR during different phases of the cell cycle. The results showed that TCR does not vary substantially during different phases of the cell cycle. More recent work involves measuring of cell cycle progression and apoptosis (programmed cell death) after UV exposure of mutant CHO cells that are deficient in different DNA repair pathways. This work showed that accumulation of DNA damage leads to G2 arrest. An important question regards the nature of the signal induced by the DNA damage.

p21 and DNA Repair: p21 is a protein that inhibits cyclin-dependent kinases, which in turn regulate the cell-cycle transitions from G1 to S and from G2 to M. p21 appears to be up-regulated in senescent cells. By inhibiting the action of cyclin-dependent kinases, p21 blocks progression through the cell cycle. Transcription of the gene encoding p21 is induced by DNA damage. This induction by DNA damage occurs by mechanisms that are dependent or independent of tumor suppressor p53. Furthermore, p21 is also directly involved in replication via its binding to proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase. PCNA is involved both in DNA replication and in DNA repair. We are therefore interested in the question of whether p21 plays a role in DNA repair.
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Recent Publications:

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Evans MK, et al. *Oncogene* 1996; 12: 651-658.

Biography: Dr. Michele K. Evans received her medical degree from the University of Medicine and Dentistry of New Jersey-The Robert Wood Johnson Medical School. She is board certified in both Internal Medicine and Medical Oncology having trained in Medicine at Emory University Affiliated Hospitals and in the Clinical Oncology Program of the National Cancer Institute (NCI). Interest in human cancer-prone disorders and DNA repair has led her to study the role of DNA repair in cancer susceptibility and aging as Senior Clinical Investigator in the Laboratory of Molecular Pharmacology, NCI and currently as a tenure track investigator at the Laboratory of Molecular Genetics, NIA.

Research: Tumorigenesis is facilitated by loss of fidelity in the replicative mechanism, accumulation of genetic lesions, and faulty DNA repair mechanisms. Similarly, aging or cellular senescence is characterized by random accumulation of damage or mutation in DNA, RNA, or proteins and perhaps a diminished ability to repair DNA. The increased incidence of cancer as a function of age underscores the mechanistic relatedness of these two cellular processes. The diminished ability to repair DNA appears to be the crucial and convergent factor. However, the consequences are distinct in cancer and aging. The overall thrust of our work has been to understand the role of DNA repair in cellular senescence and cancer susceptibility using heritable human syndromes and tumor cell lines as model systems.

DNA Repair and Cancer: Our study of the cancer prone disorder, Xeroderma Pigmentosum (XP), described DNA repair phenotypes for four complementation groups of the syndrome based on analysis of gene and strand-specific DNA repair capacity. Clinical manifestations were also assessed in terms of gene-specific repair capacity resulting in identification of a link between defective transcription coupled repair and acute sun sensitivity. We also confirmed a repair phenotype in XP complementation group C (XPC) in which active genes are repaired despite low levels of bulk DNA repair.

Because mutations in tumor suppressor genes have been implicated in many cancers, we investigated DNA repair characteristics in highly skin cancer prone XPC patients. We found that efficient repair of the *p53* gene is important in UV-induced skin tumorigenesis in XP because defective DNA repair at the gene level directly contributes to cellular transformation in the skin of these patients. These findings also led us to conclude that *p53* may be directly involved in nucleotide excision repair and that heritable syndromes associated with mutations in *p53* may have defective DNA repair pathways. This was explored by study of the Li-Fraumeni Syndrome that is characterized by germ line mutations of *p53* (in most patients), and cancer susceptibility. We were the first to report that nucleotide excision repair is defective in this syndrome. This study also showed that *p53* interacts with XPD, XPB, and CSB; proteins whose association with the transcription factor IIH complex (TFIIH) is indispensable for nucleotide excision repair activity. Our work suggests that p53 may play a direct role in nucleotide excision repair and that mutations in the p53 gene may result in defects of DNA repair that are clinically manifested by cancer susceptibility.

DNA Repair and Aging: The accumulation of unrepaired damage to DNA contributes to cellular senescence. DNA repair efficiency may decline in normal human aging. We are studying DNA repair pathways and transcription in cells from patients with segmental progeroid disorders (Werner's syndrome, Hutchinson Gilford syndrome, Rothmund Thomson syndrome) to identify which specific repair pathway may be defective and how alterations in repair and transcription can lead to premature cellular senescence. We studied the ability of Werner's syndrome (WS) lymphoblasts to repair UV-induced DNA damage of transcriptionally active and inactive genes. Gene-specific and strand-specific repair were found to be deficient in the WS lymphoblasts compared with normal controls. However, we found that repair is normal in primary fibroblast cultures from another WS patient. These results do suggest that there is abnormal gene repair in some WS lines. The phenotype is characterized by a milder defect in repair of active genomic regions than seen in another segmental progeroid disorder which we have also studied, Cockayne's syndrome. Because the repair phenotype differs slightly among the WS patients we studied, it is important to determine the mutational status of the Werner's syndrome gene (*WRN*) in these individuals. Different mutations in this gene could affect the function of the gene product a putative helicase in different ways. We are correlating the currently available information about the *WRN* mutations present in the studied patients with their DNA repair characteristics.

Breast Cancer and DNA Repair: Since breast cancer is predominately a disease of older women, if DNA repair declines as a function of age in all tissues, this would result in the accumulation of both environmental and endogenous DNA damage. Data suggesting that DNA damaging agents produce mutagenic lesions in exposed breast tissue implies that defective DNA repair of these lesions may be an early step in breast tumorigenesis. This idea is further supported by our own work and that of others suggesting that diminished DNA repair capacity may be an important risk factor in heritable and sporadic breast cancer. There is currently little available knowledge concerning the role of specific forms of DNA damage or the proficiency of specific repair pathways in breast cancer susceptibility and progression. We have begun to characterize the proficiency of DNA repair mechanisms required to remove mutagenic lesions from human breast tissue.

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Winter DB, et al. *Current Topics Microbiol Immunol* 1998; 229: 1-10.

Biography: Dr. Patricia Gearhart received her Ph.D. from the University of Pennsylvania in 1974. She performed postdoctoral training at the Johns Hopkins University and was a staff associate at the Carnegie Institution of Washington until 1982. She then became a faculty member at the Johns Hopkins University until 1995 when she moved to her present position in the Laboratory of Molecular Genetics, NIA.

Somatic Hypermutation of Immunoglobulin Variable Genes: Somatic hypermutation of variable (V) genes, which encode a portion of immunoglobulin molecules, occurs at a frequency that is a million times greater than mutation in other genes. The molecular mechanism that introduces these mutations is unknown. Our project has three aims.

Mismatch Repair and Hypermutation: The first goal is to study the mechanism that causes hypermutation of the V region. It is likely that a polymerase introduces substitutions into the DNA strands during replication or repair. If so, then immediately following hypermutation, mismatched base pairs will be present along the sequence. The mismatch repair pathway includes several proteins that recognize mismatched base pairs, excise them, and resynthesize the DNA. Thus, the mismatch repair pathway may attempt to remove some of the hypermutations before they are replicated into both strands. This predicts that the type and distribution of mutations will be different in V genes from mice defective in genes for mismatch repair. By removing the veil of mismatch repair, it may be possible to see what the original pattern of hypermutation looks like before repair acts on the mutations. We studied hypermutation in V genes from mice deficient for one of the repair proteins, PMS2, and found that although the frequency of mutation was similar to wild-type mice, the pattern was altered. In the mutant mice, there was an accumulation of adjacent base substitutions, and less bias for some types of mutation to appear on one of the two DNA strands. The data suggest that (1) tandem mutations are generated at a high frequency by a polymerase during a single event, and (2) mutations are introduced into both strands of DNA and then preferentially removed from one strand during mismatch repair. We are now examining the pattern of mutation in mice deficient for other proteins in the mismatch repair pathway, which so far has revealed novel findings about the hypermutation mechanism.

Localization of Hypermutation to the V Gene: The second goal is to identify DNA sequences around the V gene that activate the hypermutation mechanism. The concentration of mutations in a two-kb region surrounding the rearranged V gene implies that *cis* DNA sequences act as signals to target the mechanism. We propose that hypermutation is localized to the V region because the DNA sequences form stem-loop structures during transcription. Single-stranded DNA in the loops is then cut by a single-stranded endonuclease. The loops resolve back into a duplex, the nicks are made into short gaps by an exonuclease, and a DNA polymerase fills in the gaps. The resynthesis step is error-prone and introduces mutations. Mismatch repair attempts to remove the mismatches but is overwhelmed by the large number of mutations and leaves some behind, which are then replicated into both strands. This model makes the prediction that the DNA sequence around the V gene can form stable cruciforms; we are currently testing for these structures using several biochemical techniques.

Hypermutation in Old Humans: The third goal is to analyze hypermutation in V genes from old humans. As described above, we have recently correlated several patterns of hypermutation with different proteins in the mismatch repair pathway. By studying the frequency and pattern of hypermutation in old people, it will be possible to determine if the hypermutation and/or mismatch repair pathways have decreased. Genes are cloned from RNA made from peripheral blood lymphocytes taken from old and young humans, and are sequenced to identify mutations. The results will indicate if there is an age-related decline in the hypermutation mechanism, which will produce a lower number of mutations per gene, and in the mismatch repair mechanism, which will produce a different pattern of mutations.

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Recent Publications:

Filburn CR, et al. *Mech Age Develop* 1996; 87: 35-46.

Hansford RG, et al. *J Bioenerg Biomembr* 1997; 29: 89-95.

Moyes CD, et al. *Am J Physiol* 1997; 272: C1345-C1351.

Biography: Dr. Richard Hansford received his Ph.D. from the University of Bristol, United Kingdom, in 1968. After a postdoctoral fellowship with Dr. Albert Lehninger at Johns Hopkins University and a period as a lecturer at the University of Wales in Cardiff, he joined the NIH in 1973. Dr. Hansford and his group have worked mainly on the control of mitochondrial metabolism. Their move to the Laboratory of Molecular Genetics in 1996 reflected an increased emphasis on changes in mitochondrial DNA occurring in aging and implications for energy transduction.

Production of Reactive Oxygen Species by Mitochondria: The reactive oxygen species (ROS) theory of aging maintains that the balance between the rate of formation of ROS and the rate of their removal tips in favor of formation with aging, such that oxidative damage becomes cumulative. As the majority of ROS are generated by mitochondria in most cell types, the mitochondrion becomes a likely target for oxidative damage. Oxidation of mt-DNA has been suggested to be particularly important, as it may lead to mutations which may be transmitted to subsequent generations of mitochondria. Several of us in LMG are beginning to critique different aspects of this hypothesis. We have confirmed literature findings of rates of H_2O_2 formation of approximately 1% of total electron transport, when the respiratory substrate is succinate at 5mM or higher. However, physiologically appropriate concentrations of succinate or the alternative substrates pyruvate, glutamate or palmitoylcarnitine give limitingly low rates. Thus we would conclude that literature findings using saturating succinate concentrations should be evaluated with caution. We have sought to identify the parameters that make high succinate concentrations so peculiarly effective in ROS generation. Evidently, both a very high degree of reduction of Complex 1 (NADH dehydrogenase), which we identified as the radical generator in inhibitor studies, and a very high mitochondrial membrane potential are needed. We could not reproduce the literature finding of increased activity of H_2O_2 formation by heart mitochondria in senescence. However, oxidative stress is the result of formation and removal of ROS, either (or neither) of which might be altered in aging. The only way to determine the degree of oxidative damage is to measure it, and we have embarked upon such studies (see below).

Measurement of Oxidative Damage to mt-DNA and Rates of Repair: also Incidence of Deletions: The model of aging of post-mitotic tissues hypothesizes that oxidation of the bases of mt-DNA leads to mutations and deletions which in turn limit the ability to maintain cellular adenine nucleotide phosphate potentials. There are several gaps in this model. One is that the only oxidized base product of mt-DNA which has been measured is 8-oxodG, and estimates of its fractional occurrence vary over three orders of magnitude. Another missing piece in the argument is that, although some correlation between 8-oxodG levels and the so-called "common deletion" of mt-DNA has been established, it is not known that there is a cause-and-effect relationship. Finally, it has not been shown in a normal aging tissue that the incidence of deletions of mt-DNA actually rises to the point that energy transduction is compromised. Quantitative arguments become difficult as it has been suggested that deletions show a mosaic distribution amongst cells. This necessitates a cell-by-cell examination of both mt-DNA deletion burden and some index of mitochondrial functioning. This is now being approached, in a project on human T cells. Specifically, we have begun to measure the products of base oxidation in mt-DNA prepared from organs of young adult and senescent rats. We are combining enzymic measurements, using fpg-glycosylase, which cleaves 8-oxodG lesions, with chemical determinations using GC/MS and HPLC/EC methods. Initial results do not substantiate the notion of a generalized increase in base oxidation with aging. In order to study mitochondrial function on a cell-by-cell basis, we are sorting T cells from blood samples of BLSA participants on the basis of mitochondrial membrane potential, as measured with the fluorescent cation JC-1. We then use PCR methods to both quantify the so-called "common 4.8 Kb deletion" and to indicate the presence of other mutations and base damage, using LC PCR. The question is whether increased mt-DNA damage correlates with lower mitochondrial membrane potential.

Chronic Regulation of Mitochondrial Structure and Function: The reason for our interest in regulation of mitochondrial activities at the level of gene expression is two-fold. First, there is a decrease in some mitochondrial enzyme activities as a function of aging, though there is by no means a generalized decline. Second, there appears to be a decreased ability to up-regulate the tissue content of mitochondria in response to work-load: this is part of the reason for the frailty of the aged. We have chosen to emphasize the enzyme cytochrome c oxidase, as we have previously shown a decrease in specific activity of this enzyme in both heart and skeletal muscle of aged rats. Cytochrome oxidase (COX) catalyzes the terminal step of electron transfer to O_2 and exerts substantial control over oxidative phosphorylation. Further, it consists of three subunits (COX I, II, III) encoded on the mitochondrial genome, as well as several encoded on nuclear DNA. The regulation of the synthesis of the protein complex thus involves the coordinated expression of the two genomes. We have shown a decrease with aging in the COX activity of rat heart mitochondria, which is associated with a decrease in the content of the mitochondrial-synthesized polypeptides COX 1 and 11. Further, we have shown decreased rates of incorporation of radiolabelled methionine into mitochondrial proteins in general, and COX 1 and 11 subunits in particular. There is also a decrease with aging in the overall rate of transcription, as measured with isolated mitochondria and radiolabelled UTP. However, Northern analysis of individual mRNA species revealed no decrease in the message for COX 11, whereas message for COX 1 was decreased. Thus, there are issues of message stability which need to be addressed. It is known that the transcription of mt-DNA is regulated, at least in part, by a factor designated MTF-1. In turn, transcription of the gene for MTF-1 may be regulated by a factor named NRF-1. This is of profound interest, as NRF-1 regulates transcription of a number of mitochondrial enzymes which are nuclear-encoded. The obvious questions are whether NRF-1 levels are lower in the aging heart, and if so, why? We are studying these issues.

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The Molecular Defect Responsible for Premature Aging of Werner's Syndrome Patients

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Webb DK, et al. *ExperCell Res* 1996; 224: 272-278.

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Orren D, et al. *Mol Biol Cell* 1997; 8: 1129-1142.

Gilchrest B, et al. *FASEB J* 1997; 1: 322-330.

Group Members: David K. Orren, Ph.D., Michele K. Evans, M.D., A.S. Balajee, Ph.D., Robert M. Brosh, Ph.D., Amrita Machwe, Ph.D, Jan O. Nehlin, Ph.D., Vilhelm A. Bohr, M.D., Ph.D.

Werner's Syndrome (WS) is a homozygous recessive disease characterized by early onset of many characteristics of normal aging, such as wrinkling of the skin, graying of the hair, cataracts, diabetes, and osteoporosis. The symptoms of WS begin to appear around the age of puberty, and most patients die before age 50. Because of the acceleration of aging in WS, the study of this disease will hopefully shed light on the degenerative processes that occur in normal aging.

Cells from WS patients grow more slowly and senesce after fewer population doublings than age-matched normal cells, possibly because these cells appear to lose the telomeric ends of their chromosomes at an accelerated rate. Telomeric shortening is an established marker for cellular senescence. In general, WS cells have a high level of genomic instability, with increased amounts of DNA deletions, insertions, and rearrangements. These effects could potentially be the result of defects in DNA repair, replication, and/or recombination, although the actual biochemical defect remains unknown. The gene that is defective in WS, the WRN gene, has recently been identified. The amino acid sequence suggests that the WRN gene is a member of a large family of helicases with the putative ability to unwind DNA or RNA duplexes. Helicases play roles in a number of DNA involving processes: transcription, replication, DNA repair and chromatin structural organization.

Our laboratory is using several avenues to identify and characterize the biochemical defect in WS cells. One approach is to compare specific DNA related activities of normal and WS cells. After treatment with certain DNA damaging agents, both the cellular sensitivity and levels of overall DNA repair in WS cells is not elevated. However, WS cells appear to have a subtle defect in transcription-coupled repair, the highly efficient removal of lesions from the transcribed strand of active genes. Moreover, a survey of overall transcription in cells from premature aging syndromes indicated that WS cells have lower transcription rates than normal cells *in vivo.* Experiments with cell extracts suggest that this decrease in transcription might be due to the presence of an inhibitor of RNA polymerase II in WS cells. Other studies are aimed at determining whether this shortfall in total transcription might represent lower transcription of particular subsets of genes. The observed inhibition of RNA polymerase II transcription might also explain the loss of transcription-coupled repair. Studies regarding the transcription and repair activities of WS cells are ongoing; in particular, the establishment of a WS cell line transfected with the normal WRN gene will allow us to assess whether these repair and transcription problems are manifestations of the primary molecular defect in WS.

Our laboratory is also examining the slow growth characteristics of WS cells, with emphasis on determining whether this truly reflects an accelerated cellular senescence. Established markers of cellular senescence such as telomere length and beta-galactosidase activity are being compared in nontransformed WS and normal cells. We also plan to determine whether and how the cell cycle is disrupted in WS cells, reflecting the earlier observation of an elongated S phase in some WS cell lines. The initiation and elongation of replication are prime targets of study in this area, as is the response of WS cells to DNA damage during ongoing replication.

With the recent cloning of the WRN gene, our laboratory has also set our sights on obtaining purified WRN protein for use in a number of basic and complex biochemical assays. Thus far, the WRN gene has been inserted into a baculovirus vector that has been transfected into insect cells, which will putatively allow overproduction and subsequent purification of significant quantities of WRN protein. Once purified to homogeneity, WRN protein will be examined for its biochemical activities and potential interactions with other proteins.

Although progress is being made, the true nature of the biochemical defect(s) in WS is still a mystery, as is the nature of the processes that occur in cellular senescence and normal human aging. Our ongoing and future studies will be directed towards elucidation of the causes of the accelerated aging phenotype in WS, with hope that this knowledge can also be applied to our current understanding of both the aging of cells and organisms in general.

Collaborators: Drs. George Martin and Junko Oshima, University of Washington, Seattle, Washington; Anni Andersen, University of Aarhus, Aarhus, Denmark, Brian Clark, University of Aarhus, Denmark.

Oxidative DNA Damage Processing

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One theory of aging holds that oxidative damage to cellular components, such as proteins, lipids and DNA, accumulates with age, leading to the cellular dysregulation that result in the process of aging experienced by the organism. We are interested in understanding how exogenous and endogenous sources of reactive species produce oxidative damage in DNA, how that damage is processed in human cells, and the effects of unrepaired damage. Reactive oxygen species produce a wide variety of products in DNA. Differences in how these lesions are processed have made the repair of oxidative damage in DNA difficult to understand. In addition to the complex chemistry of the reactions of these reactive species with DNA and the multiple pathways involved in their repair, at least two of these species also act as intracellular messengers affecting the control of cellular processes. We seek to tease apart these complexities by introducing well defined oxidative lesions into DNA in cells *in vivo* or by studying the reactions of cell extracts or purified proteins with DNA containing well defined lesions *in vitro.*

Repair of DNA Damage Induced by Photoactivated Methylene Blue By Human Whole Cell Extracts: The damage produced in double-stranded DNA by exposure to visible light in the presence of methylene blue consists almost exclusively of the lesion, 8-oxodeoxyguanosine. This lesion is also produced in significant amounts in DNA, along with many other products, by exposure to gamma irradiation or hydrogen peroxide. By examining the repair of the methylene blue-damaged DNA by proteins extracted from human cells, we have identified two pathways of repair. The kinetics of the two pathways are distinctly different, suggesting different mechanisms involved in repair. In addition, the proportion of the lesions processed by each pathway differs markedly in cell lines derived from patients with the genetic diseases, xeroderma pigmentosum-Group A and Cockayne syndrome-Group B, in comparison with a cell line from an unaffected individual. Both xeroderma pigmentosum and Cockayne syndrome cells have characteristic defects in the repair of damage induced by ultraviolet light and in the processing of other types of oxidative damage.

Age-Associated Effects in the Repair of Oxidative Damage By Human Cell Extracts: Previously, 8-oxodeoxyguanosine has been studied as the prototypical oxidative lesion in association with aging. To test whether the two pathways for the repair of oxidative damage identified in the above study are differentially affected by the process of aging, we are examining the repair capacity of extracts derived from subjects with a range of ages. From this study, we expect to determine the variation in the repair of one type of oxidative damage in the normal population and discern any age-associated effect on these pathways.

DNA Repair Defect in Alzheimer Disease: Recent work in other laboratories, using an indirect technique reflective of DNA repair capability, has suggested that cells from Alzheimer's disease patients are defective in the processing of DNA lesions induced by irradiation with fluorescent light. We are using more traditional measures of DNA repair to assess the relative repair capacity of cells from normal and Alzheimer disease patients for various types of oxidative damage.

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Transcription and DNA Repair in Cockayne Syndrome Cells

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Dianov GL, et al. *Nucleic Acids Res* 1997; 25: 3636-3642.

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Cockayne syndrome (CS) is a rare human disease characterized by arrested post-natal growth and resulted in premature aging and death. Cells from CS individuals are abnormally sensitive to killing by ultraviolet radiation as well as certain so-called UV-mimetic chemicals, such as 4-nitroquinoline-1-oxide and N-acetoxy-2-acetylaminofluorene. This cellular phenotype prompted extensive studies on the ability of CS cells to carry out nucleotide excision repair both in intact cells and in cell-free systems. Most conventional assays, including the use of a cell-free system that supports transcription-independent nucleotide excision repair, indicate no defect in CS cells. However, CS cells are defective in the enhanced rate of repair of the template (transcribed) strand relative to the coding (non-transcribed) strand of transcriptionally active genes. In recent experiments from this laboratory, we have demonstrated that mutations in the CSB gene are the cause of the transcription coupled repair defect. In hamster cells homologous to CSB, we can transfect a normal CSB gene and complement the repair defect. The mechanism of TCR in eukaryotes remains to be elucidated, and the CSB protein appears to play an important role in this process.

These observations and the discovery of the dual function of transcription factor II H (TFIIH) in transcription and DNA repair, led to the "transcriptional hypothesis" which postulates that transcription defects are the underlying basis for the pathology in some human diseases including Cockayne's Syndrome. In this laboratory, we were interested in testing this hypothesis experimentally, and we have demonstrated a reduced level of RNA polymerase II (Pol II) transcription in intact and permeabilized CS-B cells. The molecular mechanism responsible for this deficiency was further investigated in a cell-free system. We utilized an *in vitro* transcription assay and determined Pol II transcription activity in extracts prepared from different CS cell lines in comparison to extracts prepared from normal cells. We found that *in vitro* transcription in CS extracts is highly sensitive to minor damage in template DNA arising during purification. This deficiency may be complemented by transfection of a CS-B cell line with a normal CSB gene.

Studies of transcription *in vitro* in a plasmid based system demonstrate a significant transcription defect in CSB cells. This defect may be related to oxidative damage or structural changes in the DNA which somehow affect the transcription in CSB cells but not in normal cells. Experiments in intact cells also demonstrate a defect in basal transcription which can be complemented by transfection with the normal CSB gene. Further, these experiments suggest that CSB cells may have a defect in the assembly of the higher order chromatin structural organization in conjunction with transcription and DNA repair. This is supported by the observation that CSB chromatin is much more sensitive to detergent than normal chromatin.

Future Directions: Our data suggest that a defect in CS cells may be due to increased sensitivity of RNA polymerase II transcription to DNA damage or/and accumulation of some unidentified DNA damage in CS cells. The future aim is to identify a possible DNA repair deficiency and to understand the mechanism of increased RNA polymerase II transcription sensitivity to DNA damage in CS cells.

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